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09/189415

(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Attaching and Effacing Protein of Enterohemorrhagic E. Coli

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(57) 32 Claims

Canada

Title: Attaching and Effacing Protein of Enterohemorrhagic E.coli

FIELD OF THE INVENTION

The present invention relates to a DNA segment having a sequence encoding a protein associated with attaching and effacing activity of enterohemorrhagic E.coli, or an oligonucleotide fragment thereof. The DNA segment of the invention can be used to produce a protein associated with attaching and effacing activity of enterohemorrhagic E.coli or a part thereof, by culturing a transformant host cell which includes a recombinant molecule comprising a DNA segment of the invention or an oligonucleotide fragment thereof and an expression control sequence operatively linked to the DNA segment.

The DNA segment of the invention also permits selection of DNA and amino acid sequences unique to a protein associated with attaching and effacing activity of enterohemorrhagic E.coli. Novel DNA segments or proteins can thus be constructed which contain the unique DNA and amino acid sequences. The invention also relates to uses of the DNA segments encoding a protein associated with attaching and effacing activity of enterohemorrhagic E.coli, and fragments thereof, and uses of a protein associated with attaching and effacing activity of enterohemorrhagic E.coli or parts thereof.

BACKGROUND OF THE INVENTION

Enteropathogenic *Escherichia coli* (EPEC) colonize the intestine of humans (Ulshen, M., and Rollo J. (1980) N. Engl. J. Med. 302, 99-102) and experimental animals, (Tzipori, S., Robbins-Brown, R.M., Gonis, G., Hayes, J., Withers, M., and McCartney, E. (1985) Gut 26, 570-578) producing a characteristic lesion which has been called attaching and effacing (AE) (Moon, H.W., Whipp, S.C., Argenzio, R.A., Levin, M.M., and Gianella, R.A. 1983.

verotoxin-producing E.coli which are important causes of human disease such as O157:H7 from those which, although present in animals, rarely if ever cause human disease.

Jerse et al. ((1990) Proc. Natl. Acad. Sci. USA 87, 7839-7843) and Donnenberg et al. ((1990) Infect. Immun. 58, 1565-1571) have identified a gene in the EPEC strain E2348/69 which was necessary but not sufficient for the formation of the AE lesion. This gene of approximately 3kb, which was designated *eae*, encodes a 94 kilodalton outer membrane protein (Jerse, A.E. and Kaper, J.B. (1991) Abst. Ann. Am. Soc. Microbiol. B-112, p.44). It was shown that E2348/69 mutants carrying transposon TnPhoA insertions in the *eae* gene were unable to produce the AE lesion when incubated with HEp-2 cells (Donnenberg M., Calderwood, S., Donohue-Rolfe, A., Keusch, G.T., and Kaper, J. (1990) Infect. Immun. 58, 1565-1571). A 1kb *StuI-SalI* fragment which encompasses the central one third of the EPEC *eae* gene was found to hybridize with DNA isolated from bacteria of classical EPEC serogroups as well as with DNA isolated from EHEC of serogroups 026 and 0157 (Jerse, A., Yu, J., Tall, B., and Kaper, J. (1990) Proc. Natl. Acad. Sci. USA 87, 7839-7843). Although both EPEC and EHEC strains cause ultrastructurally similar lesions, as shown in gnotobiotic piglets (Moon, H.W., Whipp, S.C., Argenzio, R.A., Levin, M.M., and Gianella, R.A. 1983. Infect. Immun. 41, 1340-1351; Tzipori, S., Wachsmuth, I.K., Chapman, C., Birnor, R., Brittingham, J., Jackson, C., and Hogg, J. (1986) J. Infect. Dis. 154, 712-716) and in tissue culture cells (Knutton, S., Baldwin, T., Williams, P.H., and McNeish, A.S. (1989) Infect. Immun. 57, 1290-1298), the two types of infection can be differentiated by anatomic site of involvement, suggesting differences in adherence factors.

SUMMARY OF THE INVENTION

The present invention provides a purified and isolated DNA segment having a sequence which codes for a protein

The invention still further provides a substantially pure protein associated with attaching and effacing activity of enterohemorrhagic E.coli in a preferred embodiment the protein has an amino acid sequence which has substantial homology with the amino acid sequence as shown in the Sequence Listing as SEQ ID NO:1

The invention also relates to antibodies specific for an epitope of a protein of the invention, preferably a monoclonal antibody, and a method for preparing the antibodies

A diagnostic kit for detecting enterohemorrhagic E.coli, preferably E.coli 0157 in a sample comprising a monoclonal antibody of the invention and directions for its use is also provided.

The invention also contemplates an immunoassay for the detection of enterohemorrhagic E.coli, preferably E.coli 0157, comprising contacting a sample suspected of containing enterohemorrhagic E.coli with a monoclonal antibody of the invention to form an immune complex; and determining the presence of the immune complex.

The DNA segments of the invention or oligonucleotide fragments of the DNA segments, allow those skilled in the art to construct nucleotide probes for use in the detection of nucleotide sequences in samples such as biological or food samples. The nucleotide probes may be used to detect nucleotide sequences that encode proteins related to or analogous to the attaching and effacing protein of the invention.

Accordingly, the invention provides a method for detecting the presence of a DNA segment having a sequence encoding a protein related to or analogous to a protein associated with attaching and effacing activity of enterohemorrhagic E.coli or an oligonucleotide fragment thereof in a sample,

The invention further relates to a kit for determining the presence of a DNA segment having a sequence encoding a protein associated with attaching and effacing activity of enterohemorrhagic E.coli or a predetermined oligonucleotide fragment thereof in a sample, comprising primers which are capable of amplifying the DNA segment or the predetermined oligonucleotide fragment thereof in a polymerase chain reaction to form amplified sequences, means for assaying the amplified sequences, and directions for its use.

The present inventors have designed specific nucleotide sequences for detecting pathogenic verotoxin-producing E.coli strains. The present invention therefore further provides a purified and isolated nucleotide sequence comprising the sequence shown in the Sequence Listing as SEQ ID NO:2; the sequence shown in the Sequence Listing as SEQ ID NO:3; the sequence shown in the Sequence Listing as SEQ ID NO:4, the sequence shown in the Sequence Listing as SEQ ID NO:5; the sequence shown in the Sequence Listing as SEQ ID NO:6; the sequence shown in the Sequence Listing as SEQ ID NO:7; the sequence shown in the Sequence Listing as SEQ ID NO:8; the sequence shown in the Sequence Listing as SEQ ID NO:9; a sequence having substantial homology thereto, or a fragment of the nucleotide sequence.

Accordingly, the present invention relates to a method for detecting verotoxin-producing E.coli of the serogroups 05, 026, 0103, 0111, 0118, 0145, and 0157 in a sample comprising contacting the sample with a SalI-StuI fragment comprising the sequence as shown in the Sequence Listing as SEQ ID NO:2 and the sequence as shown in the Sequence Listing as SEQ ID NO:3, under conditions which permit the fragment to hybridize with a complementary sequence in the sample to form a hybridization product, and assaying for the hybridization product.

SEQ ID NO:5, reagents required for hybridization of the fragment with a DNA segment encoding the attaching and effacing protein of enterohemorrhagic E.coli or an oligonucleotide fragment thereof, and directions for its use.

The invention further relates to a kit for detecting pathogenic verotoxin-producing E.coli strains of the serogroup 0157 in a sample comprising the primers P1EH as shown in the Sequence Listing as SEQ ID NO:6 and P2EH as shown in the Sequence Listing as SEQ ID NO:7, respectively, reagents required for the amplification in a PCR reaction of sequences which terminate with the nucleic acid sequence of one of the primers and the complementary nucleic acid sequence of the other of the primers, and directions for its use.

DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 shows the DNA sequence of the EHEC *eae* gene and the deduced protein sequence starting at the start codon at 206 bp;

Figure 2 shows the homology between the deduced protein sequences of the EPEC and EHEC *eae* genes;

Figure 3 shows the amino acid sequence alignment of the predicted EPEC and EHEC proteins;

Figure 4 shows the results of colony blot hybridizations of various O serogroups using A) a central (C1-C2) 1 kb fragment and B) a 3' (A3-B2) 0.5 fragment of the O157:H7 eae gene;

short stretches can be aligned. There is conservation of cysteine residues at positions 859 and 911 of the alignment.

Both the EHEC and EPEC sequences show similarity to the Yersinia pseudotuberculosis invasin gene (Isberg, R.R. et al., (1987) Cell 50, p.769) with greatest divergence at the C-terminus. The C-terminal end of the Yersinia inv gene is associated with receptor binding (Leong et al, EMBO J 1990; 6:1979-89) and it is possible that the same applies to the eae gene products.

The inventors also probed verotoxin-producing E.coli (VTEC) (251 strains) of multiple O-serogroups with a highly conserved 1kb StuI-SalI central fragment from the EHEC eae gene using colony hybridization. Only the following VTEC serogroups (125 strains) were found to be positive: O5, O26, O103, O111, O118, O145, and O157. Using the polymerase chain reaction (PCR) a less conserved 0.5kb probe was generated from the 3' end of the EHEC eae gene. Among the 251 VTEC strains tested using colony hybridization, only those strains belonging to the O157 serogroup were positive. Additionally, the 3 end of eae genes of VTEC strains appear to have unique nucleotide sequences as determined on selected O-serogroups. These O-serogroups initially hybridized with the highly conserved central regions of the eae genes.

Therefore, the present inventors have shown that the central 1 kb fragment of the EHEC eae gene is homologous in a variety of O serogroups of verotoxin-producing E.coli (VTEC) strains but that there is diversity at the C-terminal end. The heterogeneity at the 3' end of eae genes allows for specific detection of clinically relevant VTEC strains and may be associated with differences in receptor binding.

slight or inconsequential sequence variations from the sequences disclosed in the Sequence Listing as SEQ ID NO:1 i.e. the homologous sequences function in substantially the same manner to produce substantially the same polypeptides as the actual sequences. The variations may be attributable to local mutations or structural modifications.

It will also be appreciated that a double stranded nucleotide sequence comprising a DNA segment of the invention as shown in Figure 1 or an oligonucleotide fragment thereof, hydrogen bonded to a complementary nucleotide base sequence (see Sequence Listing SEQ ID NO:1), an RNA made by transcription of this doubled stranded nucleotide sequence, and an antisense strand of a DNA segment of the invention or an oligonucleotide fragment of the DNA segment, are contemplated within the scope of the invention.

A number of unique restriction sequences for restriction enzymes are incorporated in the DNA segment identified in the Sequence Listing as SEQ ID NO:1, and these provide access to nucleotide sequences which code for polypeptides unique to the protein associated with attaching and effacing activity of EHEC. DNA sequences unique to the protein associated with attaching and effacing activity of EHEC or isoforms thereof, can also be constructed by chemical synthesis and enzymatic ligation reactions carried out by procedures known in the art.

The DNA segments of the invention or oligonucleotide fragments of the DNA segments, allow those skilled in the art to construct nucleotide probes for use in the detection of nucleotide sequences in samples such as biological specimens or food samples. A nucleotide probe may be labelled with a detectable marker such as a radioactive label which provides for an adequate signal and has sufficient half-life such as ^{32}P, ^{3}H, ^{14}C or the

probe with the DNA segment or an oligonucleotide fragment thereof, and directions for its use.

The DNA segment of the invention permits the identification and isolation, or synthesis of nucleotide sequences which may be used as primers to amplify a DNA segment of the invention or an oligonucleotide sequence thereof, for example in the polymerase chain reaction (PCR). The length and bases of the primers for use in the PCR are selected so that they will hybridize to different strands of the desired sequence and at relative positions along the sequence such that an extension product synthesized from one primer when it is separated from its template can serve as a template for extension of the other primer into a nucleic acid of defined length.

The primers may be prepared using techniques known in the art such as for example phosphotriester and phosphodiester methods or automated techniques. Restriction endonuclease digests may also be used as primers.

It will be appreciated that the primers may contain non-complementary sequences provided that a sufficient amount of the primer contains a sequence which is complementary to the DNA segment of the invention or oligonucleotide sequence thereof, which is to be amplified. Restriction site linkers may also be incorporated into the primers allowing for digestion of the amplified products with the appropriate restriction enzymes facilitating cloning and sequencing of the amplified product.

It will also be appreciated that the invention includes nucleotide sequences which have substantial sequence homology with the above-mentioned nucleotide probes and primers. Further, it will be appreciated that a double stranded nucleotide sequence comprising a nucleotide probe or primer, hydrogen bonded to a complementary nucleotide base sequence, an RNA made by transcription of this

triphosphates. The detectable markers may be analyzed by restriction and electrophoretic separation or other techniques known in the art.

The conditions which may be employed in the methods of the invention using PCR are those which permit hybridization and amplification reactions to proceed in the presence of DNA in a sample and appropriate complementary hybridization primers. Conditions suitable for the polymerase chain reaction are generally known in the art. Preferably, the PCR utilizes Taq polymerase (GeneAmp Kit, Perkin Elmer Cetus) as the polymerization agent and each cycle consists of the following: denaturation at 94°C X 1 min; annealing at 55°C X 1 min; and extension at 72°C X 1 min.

The invention still further provides a kit for determining the presence of a DNA segment encoding a protein associated with attaching and effacing activity of enterohemorrhagic E.coli or a predetermined oligonucleotide fragment thereof in a sample comprising primers which are capable of amplifying the DNA segment or the predetermined oligonucleotide fragment thereof, in a polymerase chain reaction to form amplified sequences, means for detecting the amplified sequences, and directions for its use.

The present invention also relates to a method of detecting verotoxin-producing E.coli using the DNA segment of the invention and oligonucleotide fragments thereof. As hereinbefore mentioned the present inventors have found that a SalI-StuI fragment (C1-C2) comprising the sequences as shown in the Sequence Listing as SEQ ID NO:2 and SEQ ID NO:3, may be used to detect verotoxin-producing E.coli of the serogroups 05, 026, 0103, 0111, 0118, 0145, and 0157. A 0.5 kb fragment (A3-B2) comprising the sequences as shown in the Sequence Listing as SEQ ID NO:4 and SEQ ID

in the Sequence Listing as SEQ ID NO:9; a sequence having substantial homology thereto or a fragment of the nucleotide sequence. The location of the sequences of the sequence shown in the Sequence Listing as SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:7 on the O157:H7 eae gene are shown in Figure 6.

The invention relates to a method for detecting verotoxin-producing E.coli of the serogroups 05, 026, 0103, 0111, 0118, 0145, and 0157 in a sample, comprising contacting the sample with a SalI-StuI fragment (C1-C2) comprising the sequence as shown in the Sequence Listing as SEQ ID NO:2 and the sequence as shown in the Sequence Listing as SEQ ID NO:3, under conditions which permit the fragment to hybridize with a complementary sequence in the sample to form a hybridization product, and assaying for the hybridization product.

The invention relates to a method for detecting verotoxin-producing E.coli serogroup 0157 in a sample comprising contacting the sample with a 0.5 kb fragment (A3-B2) comprising the sequence as shown in the Sequence Listing as SEQ ID NO:4 and the sequence as shown in the Sequence Listing as SEQ ID NO:5, under conditions which permit the fragment to hybridize with a complementary sequence in the sample to form a hybridization product, and assaying for the hybridization product.

Hybridization conditions which may be used in the method of the invention are known in the art and are described for example in Sambrook J, Fritch EF, Maniatis T. In: Molecular Cloning, A Laboratory Manual,1989. (Nolan C, Ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. The hybridization product may be assayed using techniques known in the art. The oligonucleotide fragments may be labelled with a detectable marker as described herein and the hybridization product may be assayed by

The invention also contemplates a kit for detecting pathogenic verotoxin-producing E.coli strains of the serogroup 0157 in a sample comprising the primers P1EH and P2EH as shown in the Sequence Listing as SEQ ID NO:6 and as SEQ ID NO:7, respectively, reagents required for the amplification in a PCR reaction of sequences which terminate with the nucleic acid sequence of one of the primers and the complementary nucleic acid sequence of the other of the primers, and directions for its use.

The DNA segment of the present invention having a sequence which codes for a protein associated with attaching and effacing activity of EHEC, or an oligonucleotide fragment of the DNA segment including the nucleotide sequences of probes and primers described herein may be incorporated in a known manner into a recombinant molecule which ensures good expression of the protein or part thereof. In general, a recombinant molecule of the invention contains the DNA segment or an oligonucleotide fragment thereof of the invention and an expression control sequence operatively linked to the DNA segment or oligonucleotide fragment. The DNA segment of the invention or an oligonucleotide fragment thereof, may be incorporated into a plasmid vector, for example, pTZ18R.

The protein associated with attaching and effacing activity of EHEC or isoforms or parts thereof, may be obtained by expression in a suitable host cell using techniques known in the art. Suitable host cells include prokaryotic or eukaryotic organisms or cell lines, for example, E.coli JM 101 and E.coli LE 392. The protein or parts thereof may be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E Wansch, Vol. 15 I and II, Thieme, Stuttgart).

to Goding, J.W., Monoclonal Antibodies: Principles and Practice, 2nd Ed., Academic Press, London, 1986. In general monoclonal antibodies are prepared by obtaining hybridomas derived from immortalizing antibody producing cells obtained from a mammal having been immunized with a protein, and screening the hybridomas for production of antibody which binds the isolated protein.

The polyclonal or monoclonal antibodies may be used to detect a protein associated with attaching and effacing activity of EHEC in various biological materials or food samples, for example they may be used in an Elisa, radioimmunoassay or histochemical tests. Thus, the antibodies may be used as diagnostic reagents to quantify the amount of protein associated with attaching and effacing activity in a sample.

The monoclonal and polyclonal antibodies may be labelled with a detectable marker including various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include I^{125}, I^{131} or tritium.

The invention will be more fully understood by reference to the following examples. However, the examples are merely intended to illustrate embodiments of the invention and are not to be construed to limit the scope of the invention.

EXAMPLES

Example 1

150,53-61) was used for propagation of bacteriophage lambda GEM-11 (Promega, Madison, WI). Bacteriophage M13K07 (Rokeach, L., Haselby, J. and Ahoch, S. (1988) Proc. Nat. Acad. Sci. USA. 85, 4832-4836) was used as a helper to produce single stranded DNA from pTZ18R and recombinant derivatives.

DNA manipulation and sequencing

Plasmid DNA was prepared by the method of Birnboim and Doly (Birnboim, H.C. and Doly, J. (1979) Nucl. Acids Res. 7, 1513-1523). Bacterial genomic DNA was extracted using hexadecyltrimethyl ammonium bromide precipitation (Murray, M.G., and Thompson, W.F. (1980) Nucl. Acids Res. 8,4321-4325). Genomic DNA isolated from the EHEC strain CL8 was partially digested with *Sau*3A and fragments were size-fractionated by sucrose density gradient centrifugation (Sambrook, J., Fritch, E.F., and Maniatis, T. (1989) in Molecular Cloning, A Laboratory Manual (Nolan, C., Ed.) Cold Spring Harbor Laboratory Press, New York). The desired fragments (14-23kb) were isolated and ligated to dephosphorylated lambda Gem-11 *Bam*Hlarms (Promega, Wisconsin, MA] and packaged using the Packagene System extracts (Promega,Wisconsin, MA). Bacteriophage particles were propagated in E.coli LE392 and plated for plaque isolation.

The 1 Kb *Stu*I-*Sal*I fragment described by Jerse et al (Jerse, A., Yu, J., Tall, B., and Kaper, J. (1990) Proc. Natl. Acad. Sci. USA 87, 7839-7843) was used as a probe for the *eae gene*. It was produced by amplification of the central region of the *eae* gene of strain E2348/69 by the polymerase chain reaction (Sambrook, J., Fritch, E.F., and Maniatis, T. (1989) in Molecular Cloning, A Laboratory Manual (Nolan, C., Ed.) Cold Spring Harbor Laboratory Press, New York) using *Taq* polymerase (Perkin Cetus Elmer Corp., Norwalk, CT). Primers flanking the *Stu*I and *Sal*I sites were designed using the nucleotide sequence of the *eae* gene of E2348/69 deposited in Genbank by Jerse et al.

D4-4 with an insert of 14Kb, was chosen for further study. The fragments carrying the EHEC *eae* homologue were identified by hybridization to the 1Kb EPEC probe. The entire predicted structural gene was sequenced including 205 bp upstream and 118 bp downstream. Figure 1 shows the DNA sequence of the EHEC *eae* gene and the deduced protein sequence starting at the start codon at 206 bp. The end of the open reading frame is denoted by an asterisk. The sequence was compared with that of the EPEC *eae* gene. The EPEC and EHEC sequences are virtually identical for the first 2200 bp of the structural gene and for approximately 200 bp upstream of the start site. However, there is considerable divergence in the last 800 bp where the similarity is only 59% (Figure 2).

The amino acid sequence alignment of the predicted EPEC and EHEC proteins is shown in Figure 3. The positions of the terminal cysteine residues are denoted by asterisks. The N-terminal amino acids are highly conserved for the first 685 amino acids while for the C- terminal amino acids, only short stretches can be aligned. There is conservation of cysteine residues at positions 859 and 933 of the alignment.

Jerse et al. first noted the similarity between the central regions of *eae* gene of EPEC and the *inv* gene of *Yersinia pseudotuberculosis* (Jerse AE, Yu J, Tall BD, Kaper, J.B. Proc Natl Acad Sci USA 1990; 87:7839-43). The invasin protein, a product of the *inv* gene is an outer membrane protein of *Y. pseudotuberculosis* which has been extensively studied by Isberg et al. (Isberg, R.R., Voorhis, D.L. and Falkow, S. (1987) Cell. 50,769-778). When expressed in E.coli K12, the *inv* gene is necessary and sufficient for invasion of HEp-2 cells in tissue culture (Isberg, R.R., and Falkow, S. (1985) Nature. 317, 262-264). The invasin protein has been divided into three domains on the basis of mutational analysis and the study of fusion proteins (Isberg, R.R. (1989) Mol. Microbiol.

intestine in animal models while in humans they cause intense proximal colonic inflammation, characteristic of hemorrhagic colitis (Knutton, S., Baldwin, T., Williams, P.H., and McNeish, A.S.(1989) Infect. Immun. 57, 1290-1298; Tzipori, S., Wachsmuth, I., Smithers, J., and Jackson, C., (1988) Gastroenterology 94, 590-597; Tzipori, S., Wachsmuth, I.K., Chapman, C., Birner, R., Brittingham, J., Jackson, C., and Hogg, J. (1986) J. Infect.Dis. 154, 712-716; Riley, L. (1987) Annu. Rev. Microbiol. 41, 383-407; Karmali, M. (1989) Clin. Microbiol. Rev. 2, 15-38). Recent reports suggest that adhesions encoded by the high molecular weight plasmids of EPEC and EHEC strains are important in determining the specificity of adherence to tissue culture cells (Baldini, M.M., Kaper, J.B., Levine, M.M., Candy, D.C.A., and Moon, H.W. (1983) J. Ped. Gastroenterol. Nutr. 2,534-538; Karch, H., Hessemann, J., Laufs, R. , O'Brien, A.D., Tacket, C.O., and Levine, M.M. (1987) Infect. Immun. 55,455-461; Cantey, J.R., and Moseley, S.L. (1991) Infect. Immun. 59, 3924-3929; Jerse, A.E., Gicquelais, K.G., and Kaper, J.B. (1991) Infect.Immun. 59, 3869-3875). It remains to be seen whether differences in the C-terminal regions of the eae gene products of different EPEC and EHEC strains might result in alterations of receptor specificity.

EXAMPLE 2

Serotype Distribution of the EHEC eae Gene Among Verotoxin-Producing E.coli.

Probes based on the EHEC eae gene were used to study the serotype distribution of the eae homologue in a collection of verotoxin-producing E.coli isolated from human animal and food sources using colony hybridization. Colony hybridizations were carried out using standard techniques under highly stringent conditions (Sambrook J, Fritch EF, Maniatis T. In: Molecular cloning, A laboratory Manual,1989. (Nolan C, Ed.), Cold Spring Harbor Laboratory

characterized and serotyped isolates of verotoxin-producing E.coli and enteropathogenic E.coli strains derived from human, food and animal sources were contributed by A.Borczyk, C. Gyles and M.Karmali, The Hospital for Sick Children, Toronto, Canada. Several colonies were selected, resuspended in 50µl PCR buffer (X1) (Perkin Elmer Cetus) and boiled for 10 mins prior to use as template DNA in PCR amplifications. The PCR reaction conditions were as follows: 50µl reactions were set up using 1mM deoxynucleotide triphosphates, 1X PCR Buffer, 1 unit Taq polymerase (GeneAmp Kit, Perkin Elmer Cetus), 20 pmol of each primer and 1µl sample preparation. Each cycle (X40) consisted of the following:

denaturation @ 94°C X 1 min
annealing @ 55°C X 1 min
extension @ 72°C X 1 min.

Primers based on the O157:H7 sequence and the O111 sequence, (primers P1O and P2O) were used to amplify DNA from the various serogroups. The primers are shown in Table 1. The location of the A3, B2, P1EH, and P2EH primers on the 0157:h7 eae gene are shown in Figure 6.

Colony blot hybridization using [γ^{32}P] dATP oligonucleotide probes was used to confirm the specificity of the primers. Colony blot hybridizations were performed as described in Example 2.

Verotoxin-producing E.coli of serogroups O157 and O111 strains were detected by PCR with primers A3-B2, giving a 0.5 kb fragment. Both primer sets A3-B2 and P1EH-P2EH did not detect non verotoxin-producing E.coli of the serogroups O157 and O111. Primer set P1EH-P2EH specifically detected only O157 verotoxin positive strains giving a 0.45 kb fragment (Table 3, Figure 7). Figure 7 shows the amplification products of VTEC and EPEC using specific primer sets A3-B2 (0.5 kb) and P1EH-P2EH (0.45kb) and in particular the following:

skill in the art that changes can be made thereto without departing from the spirit and scope thereof.

Forming part of the present disclosure are the appended sequence listings.

TABLE 2

SEROGROUP DISTRIBUTION OF EAE-HOMOLOGUES AMONG VEROTOXIN-PRODUCING E.COLI

	PROBE		
	CENTRAL	3' REGION	
	C1-C2	A3-B2	P1EH-P2EH
SEROGROUP	(1.0kb)	(0.5kb)	(0.45 kb)
O157	67 / 67	18 / 18	7 / 7
O111	11 / 11	2 / 10*	0 / 5
O26	12 / 12	0 / 7	0 / 2
O5	7 / 7	0 / 6	0 / 1
O103	4 / 4	0 / 3	0 / 1
O145	5 / 5	0 / 13	-
O118	1 / 1	0 / 1	0 / 2
O113	0 / 5	0 / 4	0 / 1
O156	0 / 4	0 / 4	-
O127:H6 (E2348)	1 / 1	0 / 1	0 / 1
015:H- (RDEC1)	1 / 1	0 / 1	0 / 1

SEQUENCE LISTING

1. GENERAL INFORMATION
(a) APPLICANT: Joyce de Azavedo, James Brunton and Marie Louie
(b) TITLE OF INVENTION: Attaching and Effacing Protein of Enterohemorrhagic E.coli
(c) NUMBER OF SEQUENCES: 9

2. CORRESPONDENCE ADDRESS:
(a) ADDRESSEE: Linda M. Kurdydyk, Bereskin & Parr
(b) STREET: Box 401, 40 King Street West
(c) CITY: Toronto, Ontario
(d) COUNTRY: Canada
(e) ZIP CODE: M5H 3Y2

3. COMPUTER READABLE FORM:
(a) MEDIUM TYPE: Floppy disk
(b) COMPUTER: IBM PC compatible
(c) OPERATING SYSTEM: PC-DOS/MS-DOS
(d) SOFTWARE: DOS Text File

4. CURRENT APPLICATION DATA:
(a) APPLICATION NUMBER: Unknown
(b) FILING DATE: Unknown
(c) CLASSIFICATION: Unknown

5. ATTORNEY/AGENT INFORMATION:
(a) NAME: Linda M. Kurdydyk
(b) REGISTRATION NUMBER: 34,971
(c) REFERENCE NUMBER/DOCKET NUMBER: 3153-057/LMK

6. TELECOMMUNICATION INFORMATION:
(a) TELEPHONE: (416) 364-7311
(b) TELECOPY: (416) 361-1398

7. INFORMATION FOR SEQ ID NO:1:

(a) SEQUENCE CHARACTERISTICS:
(i) LENGTH: 3131 base pairs
(ii) TYPE:nucleic acid
(iii) STRANDEDNESS: double
(iv) TOPOLOGY: linear

(b) MOLECULAR TYPE: DNA (genomic)

(c) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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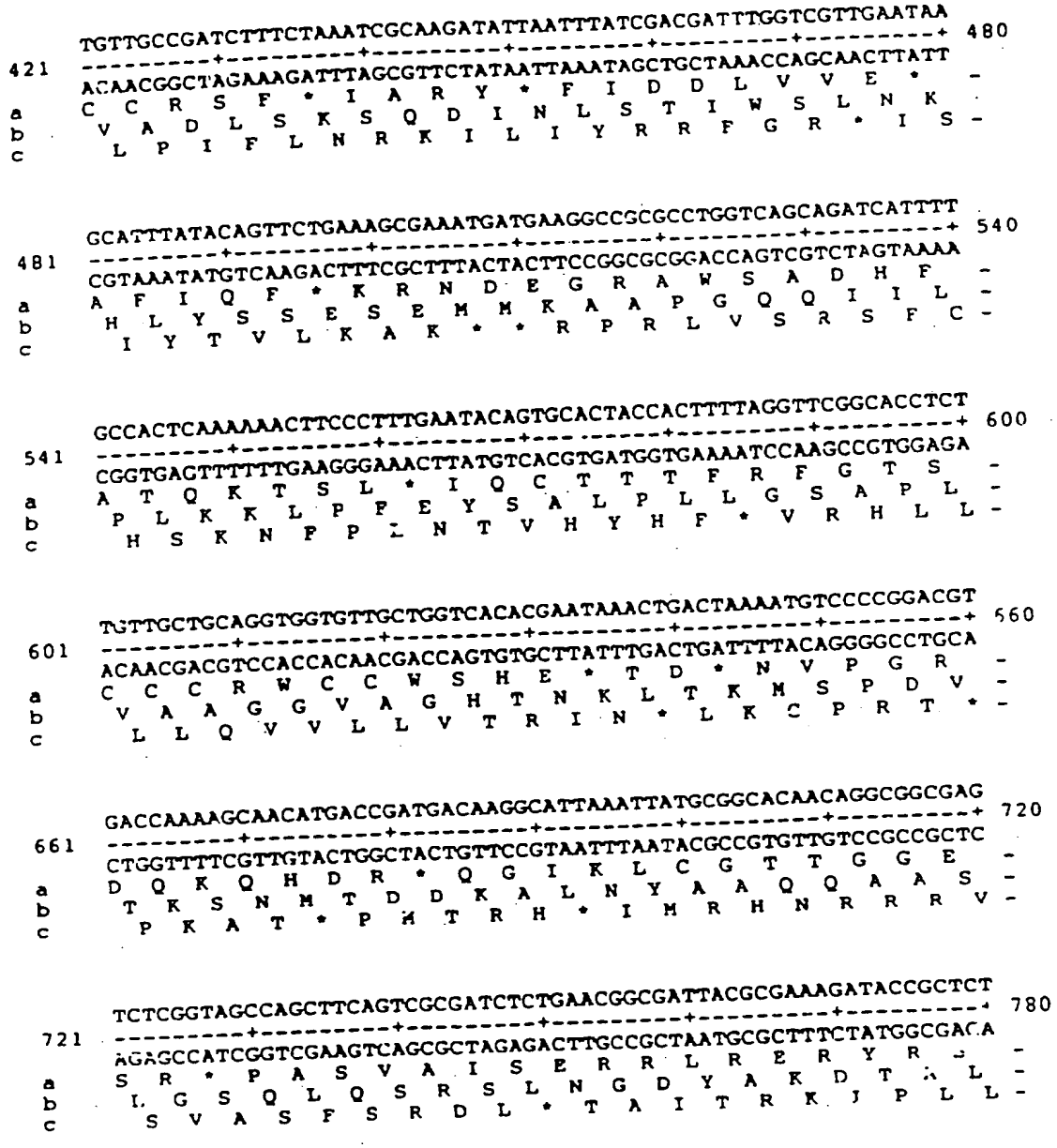
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b V A D L S K S Q D I N L S T I W S L N K -
c L P I F L N R K I L I Y R R F G R * I S -
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481
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a A F I Q F * K R N D E G R A W S A D H F -
b H L Y S S E S E M M K A A P G Q Q I I L -
c I Y T V L K A K * * R P R L V S R S F C -
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541
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b P L K K L P F E Y S A L P L L G S A P L -
c H S K N F P L N T V H Y H F * V R H L L -
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601
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a C C C R W C C W S H E * T D * N V P G R -
b V A A G G V A G H T N K L T K M S P D V -
c L L Q V V L L V T R I N * L K C P R T * -
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a D Q K Q H D R * Q G I K L C G T T G G E -
b T K S N M T D D K A L N Y A A Q Q A A S -
c P K A T * P M T R H * I M R H N R R R V -
TCTCGGTAGCCAGCTTCAGTCGCGATCTCTGAACGGCGATTACGCGAAAGATACCGCTCT 780
721
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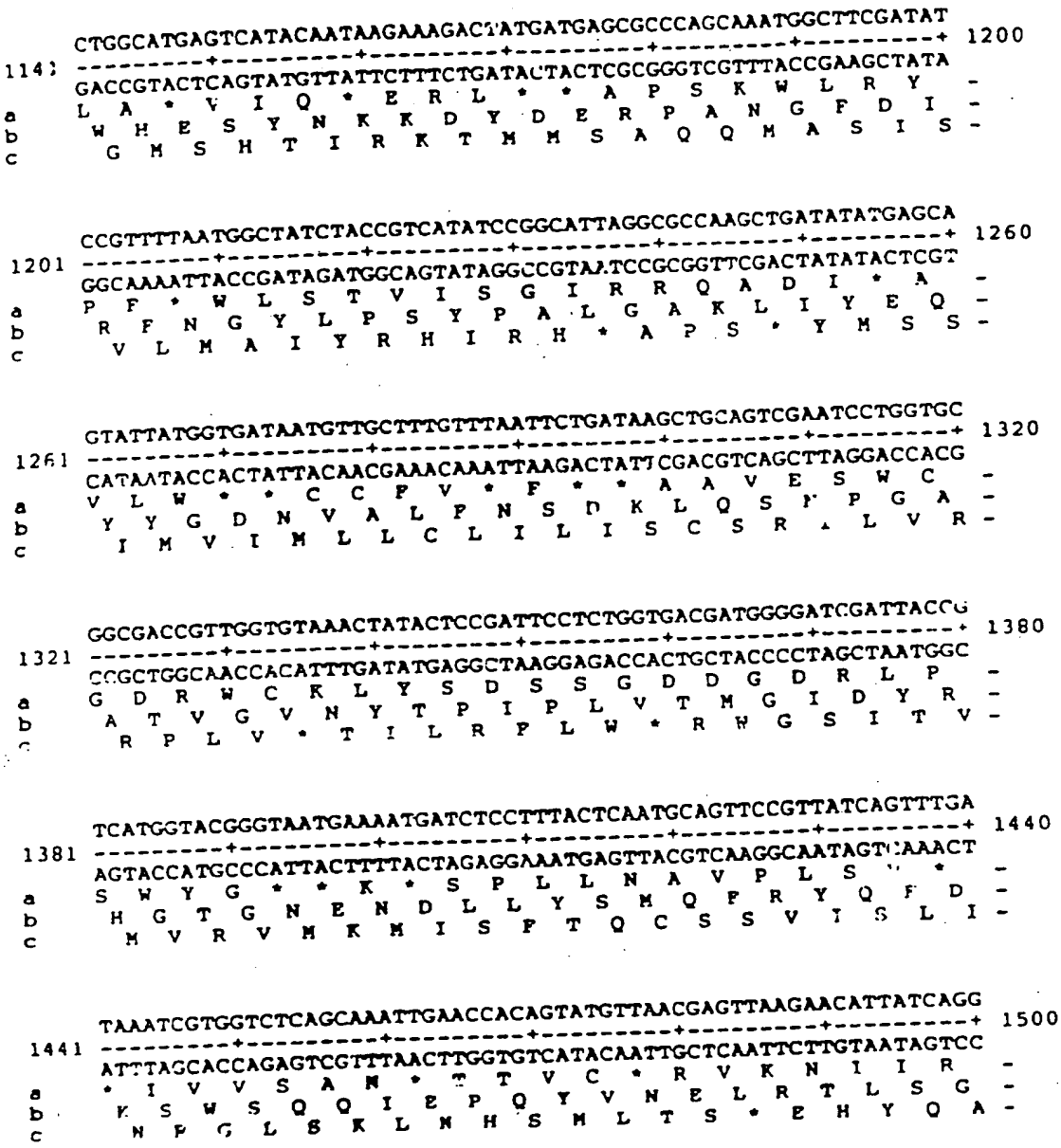
a S R * P A S V A I S E R R L R E R Y R * -
b L G S Q L Q S R S L N G D Y A K D T K L -
c S V A S F S R D L * T A I T R K I P L L -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W H E S Y N K K D Y D E R P A N G F D I -
G M S H T I R K T M M S A Q Q M A S I S -
CCGTTTTAATGGCTATCTACCGTCATATCCGGCATTAGGCGCCAAGCTGATATATGAGCA 1260
1201
GGCAAAATTACCGATAGATGGCAGTATAGGCCGTAATCCGCGGTTCGACTATATACTCGT
P F * W L S T V I S G I R R Q A D I * A -
R F N G Y L P S Y P A L G A K L I Y E Q -
V L M A I Y R H I R H * A P S * Y M S S -
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1261
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Y Y G D N V A L F N S D K L Q S F P G A -
I M V I M L L C L I L I S C S R * L V R -
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1321
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G D R W C K L Y S D S S G D D G D R L P -
A T V G V N Y T P I P L V T M G I D Y R -
R P L V * T I L R F L W * R W G S I T V -
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1381
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S W Y G * * K * S P L L N A V P L S * * -
H G T G N E N D L L Y S M Q F R Y Q F D -
M V R V M K M I S F T Q C S S V I S L I -
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1441
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* I V V S A N * T T V C * R V K N I I R -
K S W S Q Q I E P Q Y V N E L R T L S G -
N P G L S K L N H S M L T S * E H Y Q A -

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a    S  S  C  R  P  G  W  G  N  G  L  Y  G  G  *  D  F  G  *  S  -
b     Q  V  V  D  Q  V  G  V  T  D  F  T  A  D  K  T  S  A  K  A -
c      K  L  S  T  R  L  G  *  R  T  L  R  R  I  R  L  R  L  K  R -

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1921 ---------+---------+---------+---------+---------+---------+ 1980
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a    G  *  R  R  Y  H  Y  L  Y  R  D  G  E  K  E  W  G  S  S  G  -
b     D  N  A  D  T  I  T  Y  T  A  T  V  K  K  N  G  V  A  Q  A -
c      I  T  P  I  P  L  L  I  P  R  R  *  K  R  M  G  *  L  R  L -

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1981 ---------+---------+---------+---------+---------+---------+ 2040
     ATTACAGGGACAAAGTAAATTATAACAAAGTCCTTGACGTTGAGAACCCCGTTTATCACG
a    *  C  ?  C  F  I  *  Y  C  F  R  N  C  N  S  W  G  K  *  C  -
b     N  V  P  V  S  F  N  I  V  S  G  T  A  T  L  G  A  N  S  A -
c      M  S  L  F  H  L  I  L  F  Q  E  L  Q  L  L  G  Q  I  V  P -

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2041 ---------+---------+---------+---------+---------+---------+ 2100
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a    Q  N  G  C  *  R  *  G  N  R  N  V  E  V  E  Y  A  R  T  G  -
b     K  T  D  A  N  G  K  A  T  V  T  L  K  S  S  T  P  G  Q  V -
c      K  R  M  L  T  V  R  Q  P  *  R  *  S  R  V  R  Q  D  R  S -

     CGTCGTGTCTGCTAAAAGCCGCGGAGATGACTTCAGCACTTAATGCCAGTGCGGTTATATT
2101 ---------+---------+---------+---------+---------+---------+ 2160
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a    R  R  V  C  *  N  R  G  D  D  F  S  T  *  C  Q  C  G  Y  I  -
b     V  V  S  A  K  T  A  E  M  T  S  A  L  N  A  S  A  V  I  F -
c      S  C  L  L  K  P  R  R  *  L  Q  H  L  M  P  V  R  L  Y  F -

     TTTTGATCAAACCAAGGCCAGCATTACTGAGATTAAGGCTGATAAGACAACTGCAGTAGC
2161 ---------+---------+---------+---------+---------+---------+ 2220
     AAAACTAGTTTGGTTCCGGTCGTAATGACTCTAATTCCGACTATTCTGTTGACGTCATCG
a    F  *  S  N  Q  G  Q  H  Y  *  D  *  G  *  *  D  N  C  S  S  -
b     F  D  Q  T  K  A  S  I  T  E  I  K  A  D  K  T  T  A  V  A -
c      L  I  K  P  R  P  A  L  L  R  L  R  L  I  R  Q  L  Q  *  Q -
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a    W  Y  I  F  M  V  F  R  K  Y  Q  Y  R  D  C  R  C  I  R  E  -
b     G  T  Y  S  W  Y  S  E  N  T  S  I  A  T  V  D  A  S  G  K -
c      V  H  I  H  G  I  Q  K  I  P  V  S  R  L  S  M  H  Q  G  K -

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2641 ---------+---------+---------+---------+---------+---------+ 2700
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a    S  H  F  E  W  *  R  Q  C  R  N  *  S  H  I  W  *  *  A  N  -
b     V  T  L  N  G  K  G  S  V  V  I  K  A  T  S  G  D  K  Q  T -
c      S  L  *  M  V  K  A  V  S  *  L  K  P  H  L  V  I  S  K  Q -

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2701 ---------+---------+---------+---------+---------+---------+ 2760
     TCATTCAATGTGATATTTTCGTGGCAGCATATACTATTTTCACCTATTCGTTCGGATAAT
a    S  K  L  H  Y  K  S  T  V  V  Y  D  K  S  G  *  A  S  L  L  -
b     V  S  Y  T  I  K  A  P  S  Y  M  I  K  V  D  K  Q  A  Y  Y -
c      *  V  T  L  *  K  H  R  R  I  *  *  K  W  I  S  K  P  I  M -

     TGCTGATGCTATGTCCATTTGCAAAAATTTATTACCATCCACACAGACGGTATTGTCAGA
2761 ---------+---------+---------+---------+---------+---------+ 2820
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a    C  *  C  Y  V  H  L  Q  K  F  I  T  I  H  T  D  G  I  V  R  -
b     A  D  A  M  S  I  C  K  N  L  L  P  S  T  Q  T  V  L  S  D -
c      L  M  L  C  P  F  A  K  I  Y  Y  H  P  H  R  R  Y  C  Q  I -

     TATTTATGACTCATGGGGGGCTGCAAATAAATATAGCCATTATAGTTCTATGAACTCAAT
2821 ---------+---------+---------+---------+---------+---------+ 2880
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b     I  Y  D  S  W  G  A  A  N  K  Y  S  H  Y  S  S  M  N  S  I -
c      F  M  T  H  G  G  L  Q  I  N  I  A  I  I  V  L  *  T  Q  * -

     AACTGCTTGGATTAAACAGACATCTAGTGAGCAGCGTTCTGGAGTATCAAGCACTTATAA
2881 ---------+---------+---------+---------+---------+---------+ 2940
     TTGACGAACCTAATTTGTCTGTAGATCACTCGTCGCAAGACCTCATAGTTCGTGAATATT
a    N  C  L  D  *  T  D  I  *  *  A  A  F  W  S  I  K  H  L  *  -
b     T  A  W  I  K  Q  T  S  S  E  Q  R  S  G  V  S  S  T  Y  N -
c      L  L  G  L  N  R  H  L  V  S  S  V  L  E  Y  Q  A  L  I  T -
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9. INFORMATION FOR SEQ ID NO:3:

(a) SEQUENCE CHARACTERISTICS:
(i) LENGTH: 32 base pairs
(ii) TYPE: nucleic acid
(iii) STRANDEDNESS: single
(iv) TOPOLOGY: linear

(b) MOLECULAR TYPE: DNA (genomic)

(c) SEQUENCE DESCRIPTION: SEQ ID NO:3:

5'ATG GAA TTC CGA AGT CTT ATC AGC CGT AAA GT 3'

10. INFORMATION FOR SEQ ID NO:4:

(a) SEQUENCE CHARACTERISTICS:
(i) LENGTH: 29 base pairs
(ii) TYPE: nucleic acid
(iii) STRANDEDNESS: single
(iv) TOPOLOGY: linear

(b) MOLECULAR TYPE: DNA (genomic)

(c) SEQUENCE DESCRIPTION: SEQ ID NO:4:

5'ATG GAA TTC GGA TGT TCA ACG GTA AGT CT 3'

11. INFORMATION FOR SEQ ID NO:5:

(a) SEQUENCE CHARACTERISTICS:
(i) LENGTH: 29 base pairs
(ii) TYPE: nucleic acid
(iii) STRANDEDNESS: single
(iv) TOPOLOGY: linear

(b) MOLECULAR TYPE: DNA (genomic)

(c) SEQUENCE DESCRIPTION: SEQ ID NO:5:

5'ATG GAA TTC ACA ATA CCG TCT GTG TGG AT 3'

15. INFORMATION FOR SEQ ID NO:9:

(a) SEQUENCE CHARACTERISTICS:
(i) LENGTH: 18 base pairs
(ii) TYPE: nucleic acid
(iii) STRANDEDNESS: single
(iv) TOPOLOGY: linear

(b) MOLECULAR TYPE: DNA (genomic)

(c) SEQUENCE DESCRIPTION: SEQ ID NO:8:

5' TAT TTT ATC AGC TTC AGT 3'

9. A method for preparing a protein associated with attaching and effacing activity of enterohemorrhagic E.coli utilizing a purified and isolated DNA segment as claimed in claim 2.

10. A substantially pure protein associated with attaching and effacing activity of enterohemorrhagic E.coli.

11. A substantially pure protein as claimed in claim 10 having an amino acid sequence which has substantial homology with the amino acid sequence as shown in the Sequence Listing as SEQ ID NO:1.

12. A protein encoded by the purified and isolated DNA segment as claimed in claim 2.

13. A monoclonal or polyclonal antibody specific for an epitope of a protein as claimed in claim 10.

14. An antibody as claimed in claim 13 which binds a distinct epitope in an unconserved region of the protein.

15. A purified and isolated nucleotide sequence comprising the sequence shown in the Sequence Listing as SEQ ID NO:2 or the sequence shown in the Sequence Listing as SEQ ID NO:3, or a sequence having substantial homology thereto or a fragment of the nucleotide sequence.

16. A purified and isolated nucleotide sequence comprising the sequence shown in the Sequence Listing as SEQ ID NO:4 or the sequence shown in the Sequence Listing as SEQ ID NO:5, or a sequence having substantial homology thereto or a fragment of the nucleotide sequence.

17. A purified and isolated nucleotide sequence comprising the sequence shown in the Sequence Listing as SEQ ID NO:6 or the sequence shown in the Sequence Listing as SEQ ID NO:7, or a sequence having substantial homology thereto or a fragment of the nucleotide sequence.

in a sample comprising a nucleotide probe capable of hybridizing with the DNA segment or an oligonucleotide fragment thereof, reagents required for hybridization of the nucleotide probe with the DNA segment or an oligonucleotide fragment thereof, and directions for its use.

25. A method of determining the presence of a DNA segment having a sequence encoding a protein associated with attaching and effacing activity of enterohemorrhagic E.coli or a predetermined oligonucleotide fragment thereof in a sample, comprising treating the sample with primers which are capable of amplifying the DNA segment or the predetermined oligonucleotide fragment thereof in a polymerase chain reaction to form amplified sequences, under conditions which permit the formation of amplified sequences, and assaying for amplified sequences.

26. A kit for determining the presence of a DNA segment having a sequence encoding a protein associated with attaching and effacing activity of enterohemorrhagic E.coli or a predetermined oligonucleotide fragment thereof in a sample, comprising primers which are capable of amplifying the DNA segment or the predetermined oligonucleotide fragment thereof in a polymerase chain reaction to form amplified sequences, means for assaying the amplified sequences, and directions for its use.

27. A method for detecting verotoxin-producing E.coli of the serogroups 05, 026, 0103, 0111, 0118, 0145, and 0157 in a sample comprising contacting the sample with a SalI-StuI fragment (C1-C2) comprising the sequence as shown in the Sequence Listing as SEQ ID NO:2 and the sequence as shown in the Sequence Listing as SEQ ID NO:3, under conditions which permit the fragment to hybridize with a complementary sequence in the sample to form a hybridization product, and assaying for the hybridization product.

28. A method for detecting verotoxin-producing E.coli serogroup 0157 in a sample comprising contacting the sample with a 0.5 kb fragment (A2-B2) comprising the sequence as shown in the Sequence

P1EH as shown in the Sequence Listing as SEQ ID NO:5 and P2EH as shown in the Sequence Listing as SEQ ID NO:6, reagents required for the amplification in a PCR reaction of sequences which terminate with the nucleic acid sequence of one of the primers and the complementary nucleic acid sequence of the other of the primers, means for assaying amplified sequences, and directions for its use.

FIGURE 2

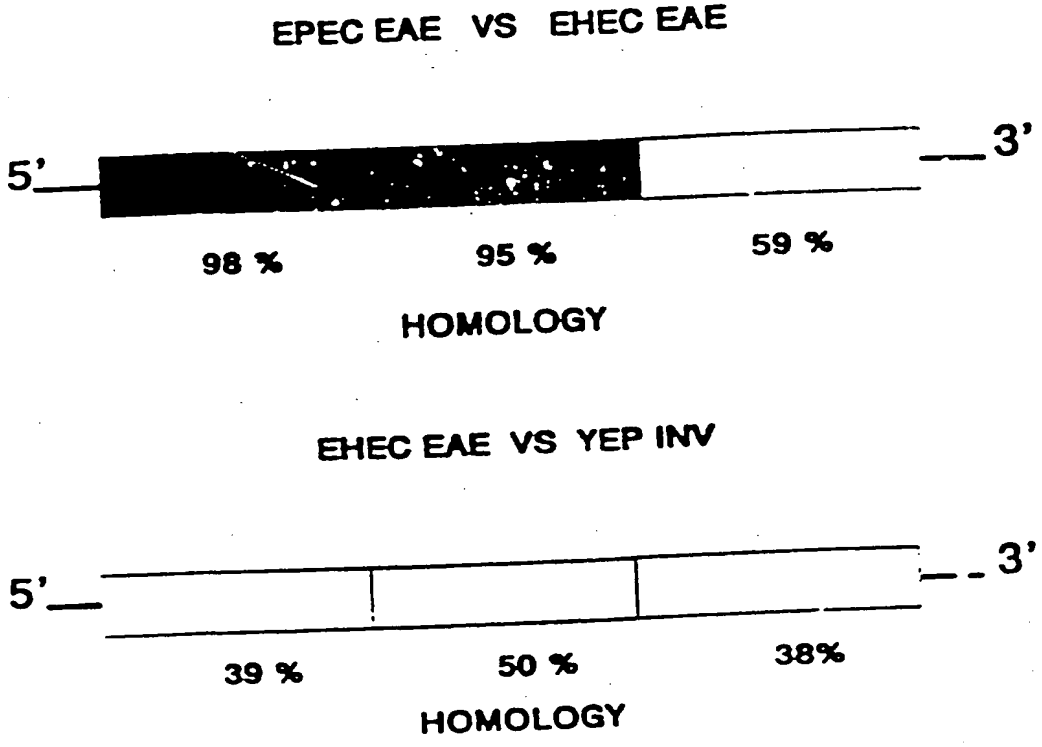

EPEC EAE VS EHEC EAE
5'
3'
98 %
95 %
59 %
HOMOLOGY
EHEC EAE VS YEP INV
5'
3'
39 %
50 %
38%
HOMOLOGY

FIGURE 4

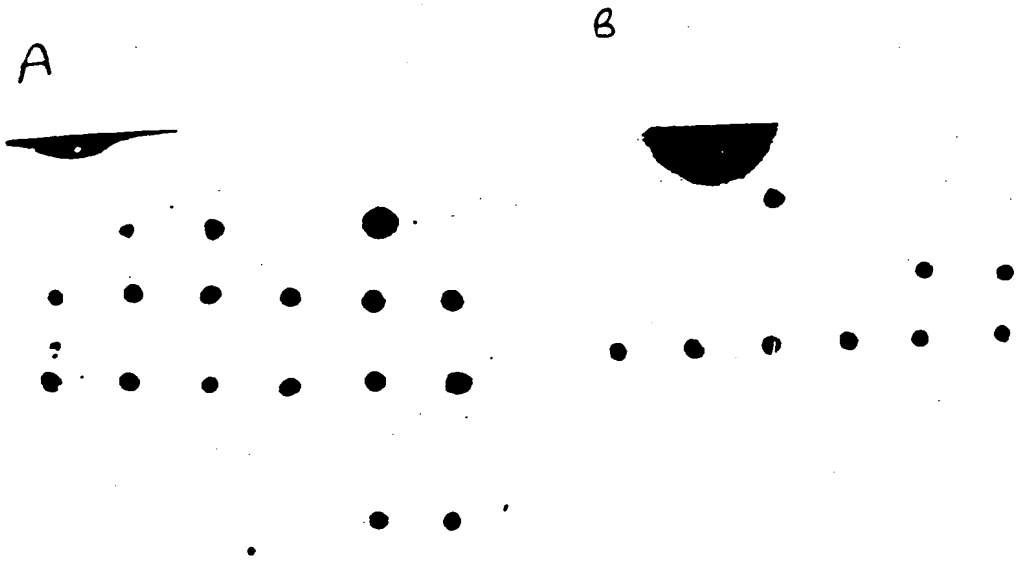
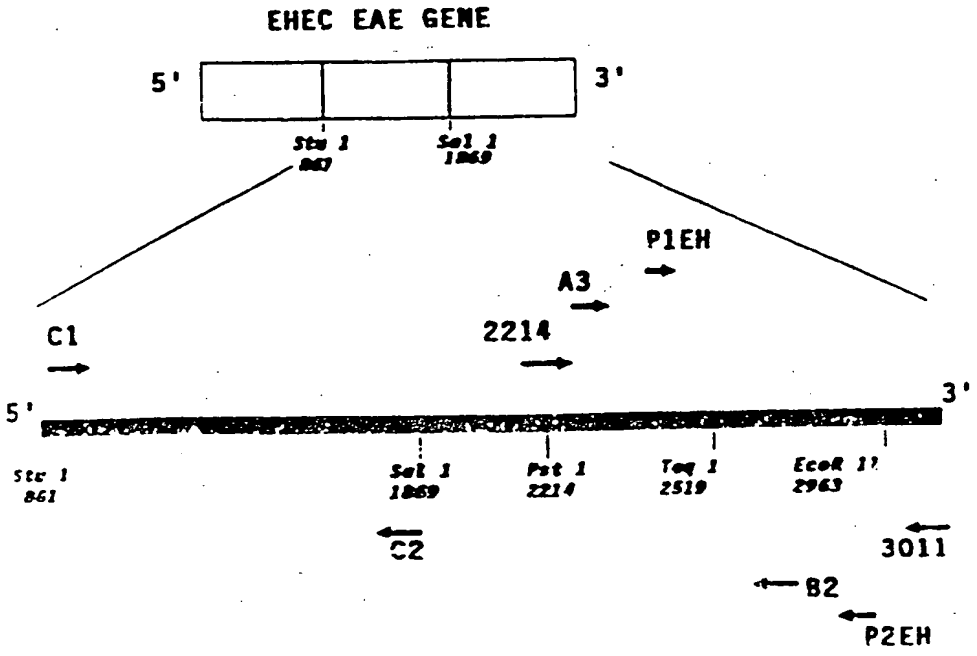


FIGURE 6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5'
3'
Stu 1
861
Sal 1
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A3
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C1
5'
3'
Stu 1
861
Sal 1
1869
Pst 1
2214
Taq 1
2519
EcoR 1?
2963
C2
3011
B2
P2EH

FIGURE 8